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PHENOLIC COMPOUNDS OF THREE UNCONVENTIONAL SUDANESE OILS

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ABSTRACT

Background. The total amount and content of phenolic and flavonoid compounds using the Folin-Ciocalteu and Aluminum chloride methods of the methanolic extracts of *Sclerocarya birrea* oil (SCO), Melon bug oil (MBO), and Sorghum bug oil (SBO) were studied.
Material and methods. Dry samples of *Sclerocarya birrea*, *Aspongopus vidiuatus* and *Agonoscelis pubescens* were used in this study. The oil was extracted using *n*-hexane following AOCS method. The phenolic compounds were extracted following a well known method and the total amounts of phenolic and flavonoids were determined using Folin-Ciocalteau and aluminum chloride methods, respectively and were identified by HPLC.
Results. The concentration of total phenolic compounds was determined as 3.3, 20.7 and 0.9 mg/100 g oil, in SCO, MBO and SBO, respectively, calculated as gallic acid equivalents. The polar fraction of the three oils was separated using solid phase extraction method. The variation of simple and complex oils phenols studied by high-performance liquid chromatography with diode-array detection (DAD) using sephadex eluted by acetone revealed six phenolic compounds which were identified as vanillic acid, callistephin, sinapic acid, *t*-cinnamic acid, epicatechin, and luteolin in SCO, and four phenolic compounds were identified as vanillin, sinapic acid, *o*-coumaric acid, and quercetin, in SBO, while in MBO four phenolic compounds were identified as *t*-cinnamic, syringic acid, quercetin and pelargonin.
Conclusions. The phenolic compounds found in SCO, SBO, and MBO can be divided into phenolic compounds and flavonoids.

Key words: *Sclerocarya birrea*, *Aspongopus vidiuatus*, *Agonoscelis pubescens*, Diol Solid Phase Extraction, Sephadex LH-20, HPLC/DAD

INTRODUCTION

Phenolic compounds have much influence on the stability, sensory and nutritional characteristics of the product and may prevent deterioration through quenching of radical reactions responsible for lipid oxidation (Ruth et al., 2001; Koski et al., 2003). Many studies investigated natural sources of phenolic

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antioxidants, and special attention is focused on the isolation of natural phenolic antioxidants from inexpensive sources. They contain phenolic compounds of various chemical natures, such as tocopherols, carotenoids, flavonoids, lignans, lignins, phenolic acids and tannins (Schmidt et al., 2003). Currently used synthetic antioxidants have been suspected to cause or promote negative health effects and there is a trend to substitute them with naturally occurring antioxidants (Fogliano et al., 1999). Among the various vegetable oils, olive, sesame (Kamal-Eldin et al., 1998), and flaxseed oils (Herchi et al., 2011) showed significant antioxidant capacities and health benefits. Low-pressure liquid chromatography using Sephadex LH-20 gel has been used in various studies to separate phenolic compounds that occur in wine (Kantz and Singleton, 1990) and in other natural products using diol phase (Mateos et al., 2001). The stability of olive oil to oxidation has been correlated to the total amount of phenolic components as well as to the ortho-diphenols and to selected simple phenol components.

Sclerocarya birrea seed contains more than 53.5% oil; the oil is rich oleic acid (67.2%) linoleic acid (5.9%), and 14.1% palmitic acid, with high oxidative stability (Mariod et al., 2004). Braca et al. (2003) isolated quercetin 3-O-α-L-(5-galloyl)-arabinofuranoside, and two epicatechin derivatives beside other eight known phenolic compounds from *Sclerocarya* leaves.

Aspongubus viduatus (melon bug) is widely distributed in Sudan where it is locally known as Um-buga and used in nutrition by collecting its oil. The bug contains 45% oil which contained 46.5, 3.4 and 44.2% oleic, linoleic and palmitic acids, respectively, with low amount of tocopherol 0.3 mg/100g and high oxidative stability (Mariod et al., 2004). The crude oil showed high antibacterial activities against some test species (Mustafa et al., 2008). *Agonoscelis pubescens* (Sorghum bug) commonly known in Sudan as Dura andat. It is one of the main pests of sorghum (Dura) in Sudan. The bug oil content was 60% with 40.9, 34.5 and 12.1% of oleic, linoleic and palmitic, respectively, the oil contains 34.0 mg/100 g tocopherols (Mariod et al., 2004).

In this work, determination and identification of phenolic compounds in Sclerocarya, Melon bug, and Sorghum bug oils are presented. The aim of this paper is to determine and identify the phenolic compounds in SCO, MBO, and SBO alcoholic extracts. It is the first time for MBO, and SBO oils to be studied for their phenolic compounds.

MATERIAL AND METHODS

Plant materials

Dried seeds of *Sclerocarya birrea* were collected manually from Western Sudan. *Aspongopus vidiuatus* and *Agonoscelis pubescens* were collected from Ghibaish and Rahad Agricultural Scheme Sudan, respectively.

Reagents and standards

All solvents used were of analytical grade. Methanol (HPLC grade) and chloroform, ß-carotene, linoleic acid, Folin-Ciocalteau phenol reagent and polyoxyethylenesorbitan monopalmitate (Tween 40) were obtained from Merck chemical company (Merck, Darmstadt, Germany). Standards phenolic compounds were acquired from Fluka Chemika (Büchi, Swizerland).

Lipid extraction

Dried seeds of *Sclerocarya birrea* were dehulled, crushed and ground by a grinding mill (Petra Electric, Burgau, Germany). The oil was extracted from the ground material by extraction with *n*-hexane (b.p. 50–60°C) in a Soxhlet apparatus for 6 h following the AOCS method (Official..., 1993).The obtained oil was kept in a plastic container at 4°C until further investigation.

Aspongopus vidiuatus and *Agonoscelis pubescens* bugs were stored in a tight polyethylene bag, killed by treatment with hot water for a few minutes and then sun dried (Mariod et al., 2004). After crushing using a lab mortar the oil was extracted using *n*-hexane (b.p 50–60°C) following AOCS method (Official..., 1993). The oil obtained by the extraction was stored at 4°C until further investigation.

Extraction of phenolic compounds

The phenolic compounds were extracted following the method of Tsimidou et al. (1996). In brief, 50 g oil were dissolved in 50 ml petroleum ether, then extracted three times with 30 mL of a mixture consisting of methanol:water (60:40 v/v). The three extracts were combined and the solvent was evaporated

to dryness in a rotary evaporator (Büchi, Switzerland) at 40°C.

Determination of the total amount of phenolic compounds

The total amount of phenolic compounds in the oil methanolic extract was determined following the method of Silvia et al. (1984). Dried samples (5 mg) and gallic acid as a standard were diluted in 1 ml of 60:40 acidified methanol/water (0.3% HCl). Test solutions (samples or standards) of 100 µl were added to 2.0 ml of 2% Na_2CO_3. After 2 min, 100 µl of 50% Folin-Ciocalteau reagent were added and allowed to stand at room temperature for 30 min. A blank consisted of all reagents and solvents without extracts or standard were prepared. Absorbance was measured against the blank at 750 nm by an U-2000 Spectrophotometer (Hitachi, Ltd. Tokyo, Japan). The standard solution was prepared from gallic acid in concentrations of 0.05 mg/ml to 0.4 mg/ml. The concentration of phenolic compounds in the extracts was calculated from a calibration curve of the standard, and expressed as mg gallic acid equivalents. All determinations were carried out in triplicate and mean value ±standard deviation (SD) reported.

Aluminum chloride/potassium acetate spectrophotometric method

Total flavonoid content was determined using Aluminum chloride/potassium acetate spectrophotometric method. Ten µL of extract, 60 µL of absolute methanol, 10 µL of $AlCl_3$ (10% w/v), 10 µL of potasium acetate (1 M) and 120 µL of distilled water were mixed and incubated for 30 min. The absorbance was measured immediately at 415 nm using microplate reader (Molecular Devices, Sunnyvale, USA). TFC was expressed as quercetin equivalents (QE) in milligrams per gram sample (Rice-Evans et al., 1996).

Separation of polyphenols using Sephadex LH-20 column

A simple chromatographic method using Sephadex LH-20 was used to separate and clean the phenolic compounds following Kantz and Singleton (1990) with some modifications in the column preparation. In brief, 5 g of Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden) was swelled in 95% ethanol for at least two hours and the slurry packed into small glass chromatography columns (5 mm diameter × 150 mm length). The column was left with solvent well above the top of the gel overnight, and one cm in length of a sea sand layer was added above the gel. The extracted phenolic compounds were prepared following Tsimidou et al. (1996). They were transferred in a methanol solution onto a sephadex column and eluted in fractions using ethanol. The absorption of the collected fractions was measured at 280, 350 nm respectively. When the absorption at 280 nm reached zero the solvent was changed to 50% acetone and other fractions were recollected and their absorption measured at the same wavelengths 280, 350 nm.

The collected fractions were evaporated to dryness, dissolved in 1 ml methanol and then transferred to a vial and kept at –18°C till the use for HPLC.

Determination of phenolic compounds by solid phase extraction using a diol-phase cartridge and HPLC

Samples of filtered SCO, MBO, and SBO (2.5 ±0.001 g) were weighed and dissolved in 6 ml hexane following the method of Mateos et al. (2001). A diol-bonded phase cartridge (Merck, Darmstadt, Germany) was placed in a vacuum elution apparatus and conditioned by the consecutive passing of 6 ml of methanol and 6 ml of hexane. The vacuum was then released to prevent drying of the column, and the solvent was pulled through, leaving the sample and the standard on the solid phase. The sample container was washed with 2–3 ml of hexane, which was run out of the cartridge. The sample container was washed again with 4 ml of hexane/ethyl acetate (90:10, v/v). Finally the column was eluted with 10 ml of methanol, and the solvent was evaporated in a rotary evaporator at room temperature under vacuum to dryness. The residue was diluted with 500 µl of methanol/water (1:1, v/v) at 40°C. An aliquot (20 µl) of the final colorless solution was injected into the HPLC.

Identification of phenolic compounds by HPLC

The HPLC consisted of a Merck Hitachi L6200A intelligent combined with a Gynkotek UVDAD Detector (Dionex, Idstein, Germany). The separation was achieved by a Dionex 4.6 × 250 mm C 16 (5 um) column (Dionex, Idstein, Germany) with an automatic

sampler. The elutes were detected at 280 nm at 25°C, the flow rate was 1.00 ml/min, the mobile phase used consisted of 5% acetic acid in water (A) and methanol (B) for a total running time of 50 min by using the following gradient: from 100% A – 0% B to 88% A – 12% B in 3.1 min, 88% A – 12% B in 21.9 min, 0.0% A – 100% B in 10 min, until the end of the run. The sample injection volume was 20 µL. The standard components were run at the same wavelength and conditions for comparison. Identification of the compounds was made by comparing the retention times (RT) and UV spectra with those of standards stored in a database.

Statistical analysis

Statistical analysis was performed by using analysis of variance (ANOVA) using Statistical programme Statistica version (StatSoft Inc. Tulsa, USA). All data are expressed as means ±SD.

RESULTS AND DISCUSSION

Total phenolic compounds

The amount of total phenolic compounds in the oil extracts from SCO, MBO, and SBO was determined by the Folin-Ciocalteau assay. The results of this colorimetric method, expressed as mg gallic acid equivalents are shown in Table 1. The three oil extracts contained 32.5, 206.6 and 9.5 mg/kg oil respectively; the melon bug oil had the higher total phenolic compounds ($P < 0.05$). Many researchers (Ruth et al., 2001; Herchi et al., 2011; Paiva-Martins and Gordon, 2005; Siger et al., 2008) studied the total phenolic content of phenolics extracted from conventional oils. Siger et al. (2008) reported high total phenolic content in pumpkin and hemp oils (25.0 and 24.0 mg/kg, respectively), and low (5.1 mg/kg) in grapeseed oil while they reported very low total phenolic content (not exceed 20.0 mg/kg) in soy, sunflower, rapeseed, corn, flax, rice bran oils. Parry et al. (2006) determined very high total phenolic contents ranged from 0.98 to 3.35 mg gallic acid equivalents per gram of cold-pressed onion, parsley, cardamom, mullein, and roasted pumpkin. Folin-Ciocalteu reagent measures the ability of any mixture to reduce phosphomolybdic and phosphotungstic acids to a blue complex. The presence of very easily oxidized substances, which considered as non-phenolic compounds, may also result in the formation of blue color with Folin-Ciocalteu reagent, causing an overestimation of total phenolic content (Padda and Picha, 2007). Total flavonoid is shown in Table 1. The three oils extract contained 8.32, 82.27 and 2.3 mg/g oil respectively; the melon bug oil had the higher total flavonoid with significant difference ($P < 0.05$) compared to SCO and SBO (Table 1).

Table 1. Total phenolic (TPC) and total flavonoids (TF) compounds in methanolic extract of unconventional Sudanese oils*

Oil	TPC, mg GAE/kg oil	TFC, mg QE 1 g oil
SCO	32.5 ±0.2[a]	8.32 ±1.0[a]
MBO	206.6 ±0.3[b]	82.27 ±1.7[b]
SBO	9.5 ±0.1[c]	2.34 ±0.6[c]

*All determinations were carried out in triplicate and mean value ±standard deviation (SD) reported. Means with different letters (a–c) within a column are significantly different at $p \leq 0.05$. SCO – *Sclerocarya birrea* oil, MBO – melon bug oil, SBO – sorghum bug oil.

Analysis of phenolic compounds in oil extract

Phenolic compounds have been repeatedly implicated as natural antioxidants in fruits, vegetables, and vegetable oils e.g. virgin olive oil. Some phenolics in studied samples, separated and identified by using high-performance liquid chromatography (HPLC) with DAD, are presented in Figures 1–3. Considerable variation was found in phenolic compounds of different oils.

Sephadex LH-20 is known to be an ideal medium for the fractionation of organic compounds on the basis of molecular size. In moderately polar mobile phases, separation is based on hydrogen bonding between carboxyl or phenolic hydrogens and H-bond acceptors in the gel. The relative adsorption depends on the number of phenolic hydrogens per molecule, and polymeric polphenols, such as condensed tannins, are more strongly adsorbed than monomeric phenols such as catechin. Quantitative recovery of the adsorbed phenolics can be readily achieved using the appropriate mobile phase (Paiva-Martins and Gordon, 2005).

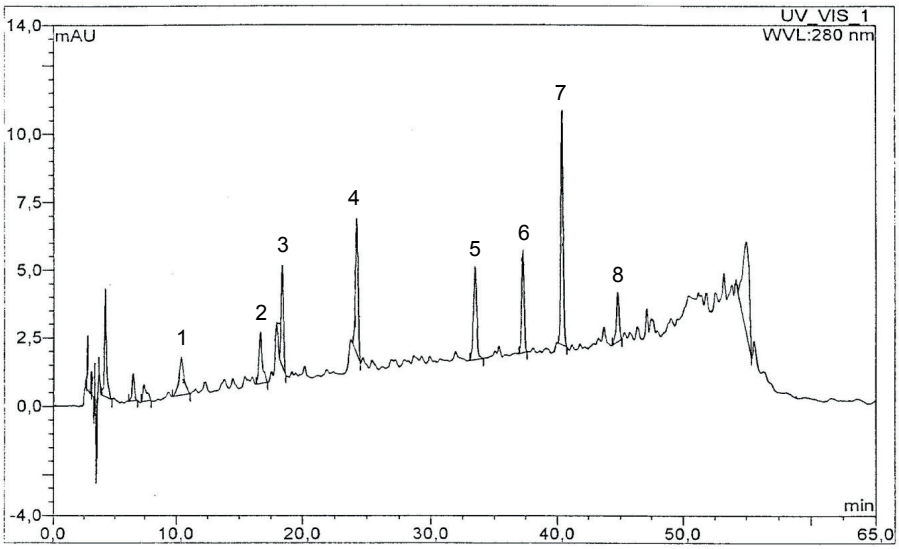

Fig. 1. HPLC chromatogram of phenolic compounds isolated from SCO using Sephadex eluted by acetone (detection at l = 280 nm). Peaks: 1 – unknown, 2 – vanillic acid, 3 – callistephin, 4 – sinapic acid, 5 – *t*-cinnamic acid, 6 – quercetin, 7 – unknown, 8 – luteolin

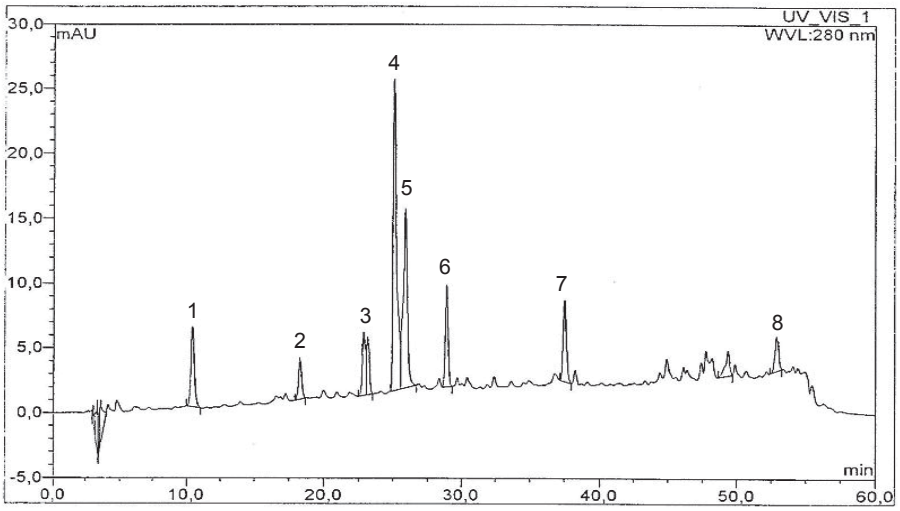

Fig. 2. HPLC chromatogram of phenolic compounds isolated from SBO using Sephadex eluted by acetone (detection at l = 280 nm). Peaks: 1 – unknown, 2 – vanillic acid, 3 – unknown, 4 – sinapic acid, 5 – unknown, 6 – *o*-coumaric acid, 7 – quercetin, 8 – unknown

The HPLC chromatogram of 22 standard phenolic compounds, which were chosen from those commonly reported in plant species were obtained. HPLC chromatograms of phenolic compounds in the methanol extract of SCO using sephadex eluted by acetone are given in Figure 1. Six phenolic compounds were

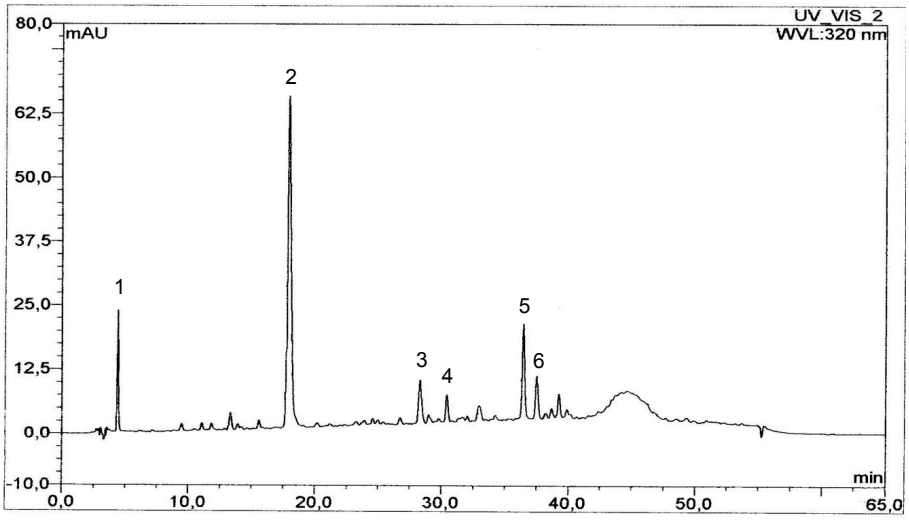

Fig. 3. HPLC chromatogram of phenolic compounds isolated from MBO by solid phase extraction (SPE) on a diol phase (detection at l = 320 nm). Peaks: 1 – unknown, 2 – syringic acid, 3 – pelargonin, 4 – unknown, 5 – *t*-cinnamic acid, 6 – quercetin

identified as vanillic acid, callistephin, sinapic acid, *t*-cinnamic, epicatechin, and luteolin, beside two unknown peaks at 10.2 and 41.5 min RT.

HPLC chromatograms of phenolic compounds in the methanol extract of SBO using sephadex eluted by acetone are given in Figure 2. Four phenolic compounds were identified as vanillin, sinapic, *o*-coumaric acid, and quercetin, besides unknown peaks. Because nonpolar phases are not appropriate for the extraction of polar fractions from nonpolar matrices, only polar phases were taken into account. Diol phase was chosen because of its negligible activity on labile (Arouma et al., 1997). The application of a diol solid phase extraction (SPE) column allowed for the extraction of polar compounds from a non polar matrix, such as oil (Herchi et al., 2011). The passing of the hexane solution of the oils through a diol cartridge retained the polar compounds on the solid phase. Hexane washing eliminated hydrocarbons, waxes, tocopherols, and triacylglycerols. Subsequent washing with hexane/ethyl acetate (90:10, v/v) removed the major part of the oxidized triacylglycerols, sterols, and diacylglycerols. The HPLC chromatogram of phenolic compounds in a methanol extract of MBO using a diol phase is given in Figure 3. The following phenolic compounds and flavonoids were identified as *t*-cinnamic acid and syringic acid, quercetin and pelargonin besides two unknown peaks at 4.5 and 30.8 min RT. Further studies for quantitative analysis of individual phenolic compounds in SCO, SBO and MBO are needed.

CONCLUSIONS

The phenolic compounds in SCO, SBO, and MBO can be divided into two groups, phenolic compounds and flavonoids. Some common phenolic acids found in SCO include vanillic acid, sinapic acid, *t*-cinnamic. Some flavonoids found in SCO include callistephin, epicatechin, and luteolin. In SBO vanillin, sinapic acid, *o*-coumaric acid, were found as common phenolic acids and quercetin as a flavonoid. In MBO only *t*-cinnamic and syringic acids, were reported as phenolic acids and quercetin and pelargonin as a flavonoid.

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